Stem cell approaches for vocal fold regeneration

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1 Abstract

Objectives:
Current interventions in the management of vocal fold (VF) dysfunction focus on conservative and surgical approaches. However, the complex structure and precise biomechanical properties of the human VF mean that these strategies have their limitations in clinical practice, and in some cases offer inadequate levels of success. Regenerative medicine is an exciting development in this field and has the potential to further enhance VF recovery beyond conventional treatments.

Our aim in this review is to discuss advances in the field of regenerative medicine; that is, advances in the process of replacing, engineering or regenerating the VF through the utilization of stem cells, with the intention of restoring normal VF structure and function.

Data sources: English literature (1946-2015) review.

Review Methods: We conducted a systematic review of MEDLINE for cases and studies of VF tissue engineering utilizing stem cells.

Results and conclusions:
The three main approaches by which regenerative medicine is currently applied to VF regeneration include cell therapy, scaffold development and the utilization of growth factors. Exciting advances have been made in stem cell biology in recent years including use of induced pluripotent stem cells. We expect such advances to be translated into the field in the forthcoming years.

Keywords: regenerative medicine; stem cells; vocal cords; tissue engineering

2 Introduction

Regenerative medicine deals with the process of replacing, engineering or regenerating human tissues with the aim of establishing normal function. Research in this field has been ongoing and successful in many different fields; it is felt that this is now the most promising approach in the treatment, or replacement, of failing tissues and organs[1,2].

Developments in the studies of VF reconstruction are gaining momentum and significantly improving our understanding of the microstructure and physiology of the VF[3]. This review will focus on the current regenerative medicine approaches used in VF reconstruction. We aim to discuss the therapies in use and under development, and summarize the ways in which the function of the VF is being most successfully restored.

2.1 Unmet clinical need for VF restoration

Loss of the laryngeal function resulting from VF dysfunction can occur secondary to a number of causes, most notably traumatic, neurological and neoplastic[4]. VF scarring is the commonest cause of poor voice following VF injury, and can be identified by the fibrotic conversion of the native extracellular matrix (ECM)[5]. Scar tissue in the superficial lamina propria (SLP) changes the tissue biomechanics of the VF as a result of increased stiffness and reduced viscosity[6], and results in a disruption of the normal mucosal wave during phonation leading to altered voice quality[4,7,8].

The negative effects, in terms of social interaction and performance at work of VF dysfunction, are frequently overlooked[9]. Voice disorders significantly affect psychosocial and physical functioning[10]. As many as 76% of patients with voice disorders are concerned about their place of employment and potential for promotion, compared to 19% of controls[11]. The management of disorders of the VFs therefore carries with it high expectations, along with associated social and professional demands[12].

Although surgical procedures are capable of repairing the current injury, they are unable to restore a native ECM composition with the necessary biomechanical properties to ensure good voice and protection against future stimuli (e.g. voice misuse and chemical irritants)[13]. Furthermore, in the management of glottic insufficiency, although VF augmentation has become popular, at present, there is no ‘ideal’ injectable material[14]. To restore normal VF function, the innate biomechanical

properties must be restored to mimic the viscoelasticity of healthy VF tissue (Figure 1). The vocal mucosa, consisting of epithelium and SLP, is the most common site for injury and scarring (Table 1). As such, it is the target for the majority of bioengineered constructs of the larynx[15].

Figure 1
Schematic diagram illustrating the various layers that comprise the vocal cord tissue micro-architecture.

Table 1
Different layers comprising vocal cord microstructure.

3 Regenerative medicine approaches

The anatomy of the VF, with its complex multilayer structure (Figure 1), makes complete restoration of the scarred or atrophied VF challenging. Currently there is no substitute for replacing diseased VFs. The principal aim of regenerative medicine is to restore the biochemical properties of the native tissue, so that the extracellular matrix can be rebuilt, and the vibratory behavior and phonatory capability of the VFs restored.

In the management of VF scarring, therapeutic options fall within one of two main principles. The first approach is to modify the wound healing process and overcome scar tissue formation. Studies have shown that injection of various materials into the injured VF have the ability to alter the post-injury inflammatory response and modify scar formation[16]. Questions still exist however as to the optimum type and timing of injection material[17]. The second approach is to provide the materials for re-building the VF once injury has already occurred. These rely on new tissue growth as opposed to modification of the inflammatory environment. Both of these approaches will be discussed later in further detail.

Regeneration of VFs requires three important elements:

- Cell therapy
- Development and implementation of a scaffold[18-21]
- Use of growth factors[22-25]

In reality, these approaches are rarely mutually exclusive with considerable overlap between them[26]. The combination of all three approaches is known as 'tissue engineering'[27]. For the purposes of this review only the use of stem cells as applied to VF tissue engineering will be discussed since scaffolds and bioactive factors have been the subject of reviews elsewhere[1]. The variety of materials available for use in VF regeneration is testament to the fact that the 'ideal' approach and material has yet to be found.

3.1 Stem cell therapy

The concept underlying cell therapy is that scarred, or atrophied, VFs will regenerate and rebuild the layers of the VF, given the correct trigger to do so. The range of cells used in cell therapy for SLP defects to date includes autologous and non-autologous mesenchymal stem cells (MSCs), fibroblasts, myoblasts, adipose-derived stem cells (ASCs), human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) (Table 2). There are two different approaches for stem cell therapy; either stimulation of endogenous stem cell populations within the VFs, or application of exogenous stem cells. These two different approaches will be discussed in the following sections.

Table 2
Potential cells sources for vocal fold regeneration.

3.1.1 Stimulating endogenous stem cell populations within the VFs

Side population (SP) cells, defined as cells that have the ability to exclude the DNA binding dye Hoechst 33342 and that contain high numbers of stem cells, have been used in the management of injured VFs[28]. An early study, designed to investigate whether SP cells exist in the human VF, found that they account for 0.2% of the total number of cells[28]. There is growing evidence that VF stellate cells found within the maculae flavae region of the VFs may include resident MSCs; the macula flavae may therefore act as a stem cell niche promoting a favorable microenvironment thereby nurturing this resident pool of stem cells[29-31].

In an attempt to investigate recruitment patterns of SP cells in the healing rat VF, unilateral VF scarring was performed in rats and immunohistochemical analysis performed 1-35 days following injury[32]. Within scarred VFs, there was a peak in the number of SP cells after 7-days, with a return to normal pre-injury levels at 14-days. The results suggested that SP cells may play a critical role in

early VF wound healing, and might therefore have therapeutic potential in the future.

Further studies have investigated human VF fibroblasts[33]. This work showed, for the first time, that these cells satisfied the definition of MSCs by possessing the appropriate cell surface markers and differentiation potential. The similarity between ASCs, bone marrow-derived MSCs and VF fibroblasts, indicates that they could all be useful in future therapies for VF repair and regeneration. However, thus far, no definitive VF stem cell has been identified that satisfies the definition of true stemness, i.e. self-renewal and multi-lineage differentiation. In addition, studies that focus on cell surface markers do not provide definitive evidence of VF stem cell populations as we currently do not know the exact surface markers of VF stem cells. In addition, even if cells express some markers known to other stem cells (such as MSCs) this does not equate to them being the same cell type.

A recent study investigated the profile of human VF fibroblasts harvested from scarred VF tissue compared to normal VF tissue[34]. The phenotypic, genotypic and protein expression properties of the VFs were examined and compared. Whilst only comparing fibroblasts from two subjects, this study provided data suggesting that fibroblasts from scarred human VFs grow significantly slower than normal VFs, but display similar morphological and contractile properties.

It is also increasingly recognized that the VF microenvironment itself is highly adapted to meet the everyday requirements of phonation. In particular, the effect of vibration has been studied to investigate whether fibroblasts and stellate cells have the ability to remodel in response to mechanical stimulation such as that experienced in the human VF[31,35-37]. This concept is being harnessed *in vitro*, in devices known as bioreactors[38,39].

Laryngeal mucosa mesenchymal stem cells (LM-MSCs) that are capable of differentiating into myofibroblasts or fibroblasts have been investigated to establish whether they could be used to improve the microenvironment in VF injury[40]. LM-MSCs from the canine epiglottis were characterized and subsequently implanted into injured canine VFs. LM-MSCs+collagen were injected intracordally and collagen alone was injected into the contralateral VF thereby serving as a control. Donor stem cell survival was demonstrated up to 8-weeks *in vivo* and cells were shown to differentiate into both fibroblasts and myofibroblasts[40]. The ability of the cells, once implanted into the VF, to regulate ECM, block collagen and decrease the inflammatory microenvironment, was proposed as an exciting technique that may be used to prevent VF scar formation. Such techniques may also be harnessed in the prevention and treatment of sulci.

3.1.2 Application of exogenous stem cells

Various cell types fall into this category including human VF fibroblasts, MSCs, ASCs, hESCs, myoblasts and iPSCs.

VF fibroblasts produce a large proportion of the ECM and are therefore essential in supporting the SLP throughout health and disease. They have therefore been the focus of many studies in an attempt to improve healing of the injured VF. Autologous fibroblasts gained from oral mucosa were first injected into the VFs of the canine model following full-thickness LP injury[41]. Videolaryngostroboscopy was used to assess VF function; performance was significantly worse at 8-weeks post-injury, but returned to near normal at 29-weeks. Histological analysis compared the injured to the uninjured VFs; an increased density of fibroblasts, collagen and reticulin were shown, with decreased levels of elastin.

More recent studies comparing three biomimetic approaches on tissue regeneration and viscoelastic properties used 20 rabbit VFs and, unlike previous studies, introduced treatment at two-months post-injury[42]. VFs were unilaterally injected with autologous fibroblasts, a semi-synthetic ECM (sECM), or autologous fibroblasts encapsulated in an sECM. The contralateral fold was injected with a saline control. All treatment groups demonstrated accelerated proliferation of the ECM, although the treatment group with autologous fibroblasts gave the best biomechanical outcomes. The use of fibroblasts embedded in sECM did not yield statistically significantly results; however, it gave better biomechanical results than the sECM-treated VF.

Unfortunately autologous VF fibroblasts, as were used in this study, remain difficult to isolate. In addition, the use of allogenic donor VF fibroblasts comes with the risk of immune rejection. Studies using more accessible fibroblasts, from the gingiva or dermis, are warranted[26].

MSCs are multipotent stromal cells characterized by specific cell surface markers and the potential to differentiate along multiple mesenchymal tissue lineages[33]. They circulate in the peripheral blood and have been shown to migrate to areas of injury, whereby they assist in tissue regeneration[43]. A prospective study using labeled MSCs (with Green Fluorescent Protein) was performed to better understand the activity of MSCs and their role in VF wound healing[43]. The results showed that circulating MSCs migrate to the site of VF injury and induce an increased expression of hepatocyte

growth factor (HGF); that is, they play a significant role in wound healing.

In vivo trials using bone marrow-derived MSCs have demonstrated some success. Cultured autologous bone marrow-derived MSCs were injected into the VFs of eight dogs, prior to injury. Results demonstrated improved VF regeneration and a reduction in scar tissue formation compared to saline control[44]. A follow-up study by the same authors determined the cell fate of the implanted MSCs, and found them to be alive, demonstrating positive expression for keratin and desmin, thereby demonstrating that they are capable of differentiating into more than one tissue type *in vivo*[45]. Kim et al[46] investigated whether mouse bone marrow-derived clonal MSCs could promote VF wound healing in the rabbit model, if injected immediately following direct mechanical injury. The treatment group showed improved morphological properties and viscoelasticity compared to the control group. Johnson et al studied the effectiveness of bone marrow-derived MSCs, either injected alone or within a synthetic ECM, on SLP regeneration[47]. The results demonstrated superiority of combination therapy to the use of bone marrow MSCs alone, since it promoted ECM deposition and growth factor production. Finally, human MSCs injected into both scarred rabbit VFs and VFs following scar excision resulted in enhanced healing and restoration of viscoelastic function[48-50]. Results demonstrated improved viscoelastic properties of the VF, with fewer signs of scarring compared to untreated VFs. The MSCs persisted for 4-weeks; a further study showed no MSCs were evident 3-months following injection. Currently, there is an ongoing clinical trial at Karolinska investigating the effects of autologous bone marrow-derived MSCs, with or without a hyaluronan gel, in patients with VF scarring. The effects on voice quality and voice function are being studied. The results will provide important information concerning the clinical effectiveness of MSCs in the management of VF scars.

ASC transplantation into localized lesions of the VF mucosa have shown promising results[51,52]. *In vitro* studies have demonstrated the secretion of several growth factors from ASCs that balance collagen and hyaluronic acid (HA) levels in the ECM[44,53]. More recently, prospective animal studies have been carried out comparing the therapeutic potential of ASCs versus MSCs when locally injected into injured rat VFs[54]. Histological and immunohistochemical results showed that VFs treated with either ASCs or MSCs had comparable regenerative outcomes. However it was concluded that since ASC infiltration resulted in a significant increase in HA, an improved anti-fibrotic effect and a more pronounced upregulation of HGF, ASCs might be superior. One such system for harvesting ASCs is the Lipogems device[55]. Direct studies comparing the Lipogems™ device (or similar systems that retain high yields of MSCs and ASCs) to conventional fat injection techniques for VF medialization are required.

ASCs embedded within HA/alginate hydrogels and injected into VFs immediately following injury revealed that ASCs injected within a hydrogel carrier produced more favorable changes than injection of ASCs alone[56]. In particular, it prevented excess deposition of type I collagen, increased HGF activity and improved VF viscoelastic properties. In conclusion, the hydrogel prolonged the retention time of the ASCs and promoted better VF wound healing.

Alternative paradigms have examined the role of hESCs in the prevention of VF scarring. One such study injected hESCs into 22 scarred rabbit VFs[57]. Results revealed significantly improved VF viscoelasticity compared to non-treated VFs. In addition, hESC-derived cells were identified within regenerating tissue and in close proximity to, or intermixed with, native tissue. These findings provide some evidence that hESCs are capable of regenerating VF tissue.

Whereas treatments discussed so far have focused on restoring the anatomical structure of VF SLP, myoblasts have the potential to restore dynamic function. Thus, autologous myoblasts have been injected into denervated rat thyroarytenoid muscles[58]. At two-months there was evidence of fusion of myoblasts with thyroarytenoid myofibers and in two specimens adductor motion was seen. The same group subsequently injected myoblasts into the denervated larynx of 20 rats, using one of four adjuvant therapies[59]. Analysis performed one-month later showed extensive stem cell survival, with fusion of cells with denervated myofibers. A further study by the same group investigated whether neurotrophic-factor secreting myoblasts could be used to selectively direct and promote re-innervation of certain laryngeal muscles following recurrent laryngeal nerve injury[60]. Results showed that ciliary neurotrophic factor strongly promoted myoblast survival and reinnervation of the denervated thyroarytenoid myofibers. Such a technique may further improve the microenvironment and help direct reinnervation. Recent studies have also demonstrated that bone marrow-derived MSCs can lead to the regeneration of functional laryngeal muscle leading to enhanced functional recovery of VF motion[61].

Finally, iPSCs, discovered in 2006[62], are gradually permeating into the field of VF regeneration[63]. iPS cells were shown capable of differentiating into non-keratinizing stratified squamous epithelial cells that may be utilized in future for VF tissue engineering[63]. The advantages of iPS are the large numbers of cells that may be generated from this technique. Direct reprogramming approaches may also have a role in the future[64].

4 Conclusions

This aim of this article was to discuss the approaches for VF reconstruction using regenerative medicine techniques. The wide variety of approaches described here is testament to the fact that this is still a very active area of research, and one that is rapidly expanding.

Numerous pre-clinical animal studies have shown promising results. However, the majority have been performed on the acutely injured VF. Treatment of *established* VF scarring is a different entity to *preventing* scar formation and further work should reflect this. In addition to this, there needs to be clinical translation to patients, with significant numbers and controls in order to gain reliable results. The structure of the human SLP is unique to humans, and clinical trials are therefore required in order to ascertain the efficacy of various interventions. Tissue biomechanics is the most meaningful outcome in the assessment of VF function in such trials, since this viscoelasticity will affect voice quality and patient outcome.

Research is ongoing in this field, and the development of new models and techniques in the management of VF reconstruction will continue to excite and aid progress in this field.

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